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SCREENING OF ORGANOPHOSPHORUS ACID ANHYDRASES FROM DIFFERENT SOURCES BY WESTERN BLOT ANALYSIS

T-C. Cheng
M. Miller
J. DeFrank

RESEARCH DIRECTORATE

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13. ABSTRACT (Maximum 200 words)

Enzymes that are capable of degrading organophosphorous compounds (e.g., acetylcholinesterase inhibitors, pesticides, and G-type agents) have been found in both procaryotic and eukaryotic organisms. These enzymes, referred to as organophosphorous acid anhydrases (OPA anhydrases), offer considerable potential for use in chemical agent decontamination systems. One of these enzymes, OPAA-2 from halophilic JD6.5, has recently been purified to homogeneity. The polyclonal and monoclonal antibodies against OPAA-2 have been prepared and shown to react specifically with this enzyme. Using this monoclonal antibody as a probe to screen OPA anhydrases from various sources in Western blot analysis, it was found that microorganisms with high levels of OPA anhydrase can be recognized by their unique binding ability. However, some cross-reaction with other unrelated proteins or enzymes may also exist. The results suggest that this monoclonal antibody can be a powerful tool for later purification, detection, and cloning of OPA anhydrases from these sources.

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PREFACE

The work described in this report was authorized under Project No. 1L162622A553, CB Defense and General Investigation. This work was started in January 1989 and completed in December 1989.

The use of trade names or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Animal Resources, National Research Council.

This report has been approved for release to the public.

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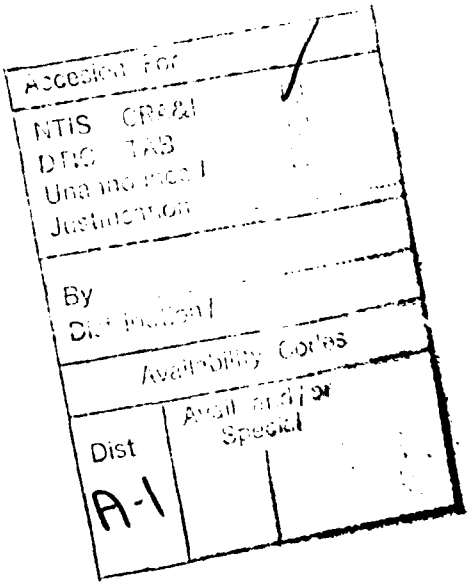

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CONTENTS

LIST OF FIGURES AND TABLES

Figures

Table

SCREENING OF ORGANOPHOSPHORUS ACID ANHYDRASES FROM DIFFERENT SOURCES BY WESTERN BLOT ANALYSIS

1. INTRODUCTION

Enzymes that are capable of degrading G-type organophosphorus agents have been found in a number of organisms, both procaryotic and eukaryotic. Previously, these enzymes were known as DFPases, somanases, paraoxonases or parathion hydrolases, based on their substrate specificity. The proliferation of both enzymes and enzyme names lead to the coining of a generic name for these enzymes, Organophosphorus Acid Anhydrases or OPA Anhydrases (First DFPase Workshop, Marine Biological Laboratory, Woods Hole, MA, June 5-6, 1987). It is planned that this name be used until the natural substrate and function of these enzymes have been identified. Diisopropyl fluorophosphate (DFP) is a common substrate used to screen for OPA Anhydrase activity.

Enzymes such as OPA Anhydrases offer several advantages for use in chemical agent decontamination systems. These catalytic enzymes can be effective under normal and extreme field conditions and are themselves non-toxic. Their reactions are fast acting and less corrosive to skin, materials and equipment. They can also be concentrated for storage and transportation. Of all the organisms that have been under investigation as sources for decontaminating enzymes, several halophilic bacterial strains isolated by Dr. DeFrank and a number of Alteromonas species obtained from the American Type Culture Collection (ATCC, Rockville, MD) have been found to have very high OPA Anhydrase activity. Some of these microorganisms possess at least two and possible several different OPA Anhydrases that have activity against DFP. Purification of these enzymes has been underway at different government agencies, industries and universities.

Recently, OPA Anhydrase-2 (OPAA-2) from Halophile JD6.5 (tentatively identified as a species of Alteromonas) has been purified to homogeneity at the U. S. Army, Chemical Research, Development and Engineering Center, Biotechnology Division (10). Polyclonal anti-serum and a monoclonal antibody against this enzyme have been prepared and shown to react specifically with the purified enzyme. The objective of this study was to use these antibodies as probes to screen OPA Anhydrases from various sources and see whether any common antigenic domains could be found. The finding of such domains could be valuable for later purification, detection and cloning of these enzymes. This study could have considerable future impact on the development of enzyme-based detoxification and detection system by the U.S. Army.

2. MATERIALS AND METHODS

2.1 Cultures.

Halophile JD species were isolated by Dr. DeFrank (11) and Alteromonas species were provided by Darrel Sledjeski and Joseph Leahy, Department of Microbiology, University of Maryland. All halophile JD species (6.5, 11.5, 26.1, 28.3 and 30.3), and A. espejiana, A. haloplanktis, A. macleodii, A nigrifaciens and A. undina were grown on HM Medium (50 g NaCl, 10 g $MgSO_4$, 10 g proteose peptone, 6 g yeast extract, 5 g casamino acids, 2.5 g HEPES per liter, pH 6.5). Alteromonas citrea, A. luteoviolaceae and A. rubra were grown on Instant Ocean Medium (38 g Instant Ocean, 5 g proteose peptone, and 1 g yeast extract per liter, pH 7.0). Alteromonas colwelliana and A. putrefaciens were grown on 278 Medium (24 g NaCl, 10 g proteose peptone, 0.7 g KCl, 7 g $MgSO_4.7H_2O$, 5.3 g $MgCl_2$, 3 g yeast extract per liter, pH 7.2). Thermophiles JD100, JD200 and JD300 were grown in a medium consisting of 2 g NaCl, 7 g yeast extract, 5 g Tryptone, 5 g Neopeptone, and 10 ml Wolin Salts Solution per liter, pH 6.8. Escherichia coli-HB101 and Yeast-TBY 747 were grown in LB Medium (12). Crude enzyme extracts containing OPA Anhydrase activity from Tetrahymena, squid, clam, hog and rabbit were gifts from Dr. Wayne Landis. Routinely, a 50 ml overnight culture of each of these strains was used to inoculate 1.5 liters of medium in 6-liter flasks. Except those thermophiles which were grown at 65°C, other strains were grown at 30°C in an incubator shaker. Cells were then harvested by centrifugation (7,500xg) for 10 minutes and stored at -20°C.

2.2 Preparation of Crude Enzyme Extracts.

Frozen, harvested cells were resuspended in 10 BM buffer (10 mM Bis-Tris Propane, 0.1 mM $MnCl_2$, pH 7.2) at a ratio of 1 g of cells for each 3 ml of buffer. The cells were disrupted by passage through a French Pressure cell (SLM Aminco) at 16,000 psi. Cellular debris was removed by centrifugation (46,000xg) for 30 minutes at 4°C. The crude cell supernatant which contained OPA Anhydrase activity, is referred to as the crude extract.

2.3 Enzyme Assay.

OPA Anhydrase activity was routinely assayed by monitoring fluoride release by an ion-specific electrode as been described in the literature (7). The reaction medium contained, 500 mM NaCl, 50 mM Bis-Tris Propane, pH 7.2, 3 mM DFP (in isopropanol), 1 mM $MnCl_2$ and 10-50 ml of enzyme solution in a total volume of 2.5 ml. Assays were routinely run at 25°C in a thermostatically controlled vessel with stirring. The enzyme sample was preincubated in the reaction medium for 1 minute before reaction

was initiated by the addition of the DFP. The reaction was monitored for 4 minutes and the rate of fluoride release corrected for spontaneous DFP hydrolysis. One unit of OPA Anhydrase activity catalyzes the release of 1.0 umole of fluoride per minute at 25°C. Specific activity is expressed as units per milligram of protein.

Hydrolysis of chromogenic substrates was conducted in an identical reaction mixture as above, with a reduction in substrate concentration to 5-100 uM. Activity was monitored by following the increase in absorbance at 405 nm (for p-nitrophenol) and units expressed as 1.0 umole p-nitrophenol released/minute. The concentration of p-nitrophenol was determined from a standard curve with authentic material.

2.4 Preparation of Polyclonal and Monoclonal Antibodies.

A Sprague-Dawley rat was immunized with HPLC purified OPAA-2 (10). The animal was given a footpad injection of 100 ug enzyme in complete adjuvant (Bacto). Three weeks later it received a subcutaneous booster of 20 ug enzyme in incomplete adjuvant (Bacto). Three days before the fusion 5 ug OPAA-2 in sterile saline was administered intravenously.

The rat was deeply anesthetized and sacrificed by exsanguination via the inferior vena cava. Serum containing polyclonal antibodies was processed from the blood and frozen in small aliquotes for future use. After the spleen was sterilely removed it was transferred to an 80 mesh Cellector tissue sieve (Bellco Glass), where the cells needed for the fusion were separated from the connective tissues of the organ.

The method described by Kennet, McKearn and Bechtol (1980), was used to fuse 3.6×10^7 immunized rat spleen cells with an equal number of Sp2/0-Ag14 mouse myeloma cells (Accession # CRL 1581; American Type Culture Collection, Rockville, MD). Following the mixing and final wash of the cells with serum free Dulbecco's minimum essential medium (DMEM), they were gently resuspended in 0.5 ml PEG 1500 solution (Boehringer Mannheim), and centrifuged at 100xg for 5 minutes. The fused cells were washed and suspended in 50 ml HAT medium [DMEM containing 1×10^{-4} M hypoxanthine, 8×10^{-7} M aminopterin, 1.6×10^{-5} M thymidine, and 20% iron supplemented bovine calf serum (Hyclone Laboratories)] and distributed in 100 ul portions into 96-well tissue culture plates at a density of approximately 1.4×10^5 cells/well.

A Rat ExtrAvidin Staining Kit (Sigma) was used in a standard enzyme linked immunoassay (ELISA) format to determine the presence of monoclonal antibody (Mab) specific for OPAA-2 in culture medium from wells containing macroscopic hybridoma colonies. Culture supernatants (50 ul) were diluted 1:1 with

10 mM phosphate buffered saline (PBS) containing 0.05% Tween 20 (Sigma) and were added to wells of microtiter plates previously coated with 10 ug/ml purified OPAA-2 in carbonate coating buffer. Biotinylated affinity purified secondary antibody to rat immunoglobulin (1:500, in PBS + 0.05%T) and avidin conjugated peroxidase (1:300, in PBS) were used sequentially to label bound Mab. Positive results were signaled by qualitative color change in the 4-chloro-1-naphthol substrate.

Populations of cells from positive supernatants were transferred to 24-well culture plates, expanded and subsequently cryopreserved for future use.

2.5 Protein Determination.

The Coomassie Protein Assay Reagent (Pierce) was used for protein determination with bovine serum albumin as the standard.

2.6 Western Blot Analysis of OPA Anhydrases.

The crude enzyme samples (150mg) were mixed with equal volume of 2x loading buffer (0.125M Tris, pH 6.8, 4% SDS, 100mM DTT, 10% glycerol and 0.025% bromophenol blue), incubated for 3 minutes at 100 C and layered on a 7.5% SDS-polyacrylamide gel (SDS-PAGE) according to Laemmli (14). The gel was run at 80 mA until the tracking dye was 1-2 cm from bottom of the gel. Prestained marker proteins (Bethesda Research Lab) were included on each gel. After electrophoresis, the polyblot transfer system (American Bionetics) was used to electrophoretically transfer the proteins in the gel onto nitrocellulose membrane. To saturate non-specific protein binding sites, the membrane was preincubated with TBS (10mM Tris, pH 8.0 containing 150mM NaCl) and 2% Bovine serum albumin (BSA) for 2 hours. After removal of the blocking buffer, a solution of either 1:1000 (v/v) polyclonal serum or 1:5 (v/v) Mab in PBS buffer was added and incubated for 2 hours. The membrane was then incubated sequentially for 1 hour with rabbit arti-rat IgG and ExtrAvidin peroxidase conjugate (Sigma) with washing in TBS-0.05% Tween-20 (TBS-T) between each incubation. After a final wash with TBS, the bound peroxidase was assayed by 30 mg 4-chloro-1-naphthol substrate (Sigma) in 50 ml TBS containing 0.03% hydrogen peroxide. When the color had developed to the desired intensity, the reaction was stopped by rinsing the membrane in two changes of deionized water.

3. RESULTS AND DISCUSSION

The polyclonal anti-serum and monoclonal antibody specific for OPAA-2 were prepared by a technically straight forward procedure (13). Rat polyclonal antibodies were raised by repeatedly boosting with the antigen (OPAA-2). For practical reasons, the monoclonal antibodies were prepared for their ability to be produced in unlimited quantities, their homogeneity

and specificity of binding. Among 6 possible clones that have been isolated, only one (Mab#6) was positively identified as specific for OPAA-2.

Halophile JD6.5, tentatively identified as a species of Alteromonas, possesses at least two OPA Anhydrase activities. Among all the OPA Anhydrases of different sources (see "Materials and Methods") that are able to hydrolyze DFP. the JD6.5 enzyme is known to have one of the highest activity. The predominant enzyme, OPAA-2 which accounts for nearly 90% of the total activity, can separated from OPA Anhydrase-1 (OPAA-1) by DEAE-Sephacel chromatography (10). OPAA-2 has been purified to homogeneity and well characterized (15).

The polyclonal anti-serum and monoclonal antibody prepared were shown to react specifically with OPAA-2. As shown in Fig. 1, the purified OPAA-2, compared with various crude fractions from JD6.5 (10), were analyzed by Western blotting after SDS polyacrylamide gel electrophoresis (SFS-PAGE). The blots were analyzed with either monoclonal antibody (A) or polyclonal anti-serum (B). A single band of purified OPAA-2 was detected in both blots (A-5, and B-1). Similar OPAA-2 band was also observed in crude extract (A-4) and OPAA-2 fractions (B-3) obtained from DEAE-Sephacel Chromatography (10). These Western blotting analyses further confirm the purification of OPAA-2 and immuno-binding specificity of Mab#6. In addition, the monoclonal antibody was shown to react with 2 other protein bands (MW 74,000 and 78,000) in crude extract and OPAA-1 fractions of DEAE-Sephacel chromatography (A-1, and B-3). In Western blots developed with polyclonal anti-serum, these two bands and another band (MW 53,000) were also detected (A-4, and B-2). These bands were much more intense than those resulting from OPAA-2, indicated that OPAA-1 possesses smaller specific activity (higher amounts of protein with less enzyme activity). Furthermore, in partially purified OPAA-1 preparation that subjected to Sephacel S-200 chromatography, the enzyme activity was found in proteins with molecular weight of 75,000. These results suggest that OPAA-1 and OPAA-2 may have some common antigenic domains.

Previously, Dr. DeFrank (11) isolated several other halophilic strains (JD11.5, JD26.1, JD28.3 and JD30.3) that possess high enzyme activities against DFP. To see whether Mab#6 can cross-react with enzymes isolated from these strains, similar Western blotting analysis was also carried out. In this experiment, equal amounts of enzyme extract from each sample was subjected to analysis. As shown in Fig. 2 (lanes 11 to 15), the blot revealed the number, molecular weight and intensity of proteins from all these strains were varied. No protein bands (data not shown) were detected in control cells (E. coli-HB101 and Yeast-DBY747) which are known to have little or no enzyme activity against DFP. Besides OPAA-2 of JD6.5, one of the OPA anhydrase activities found in JD30.3 (MW 60,000) has been

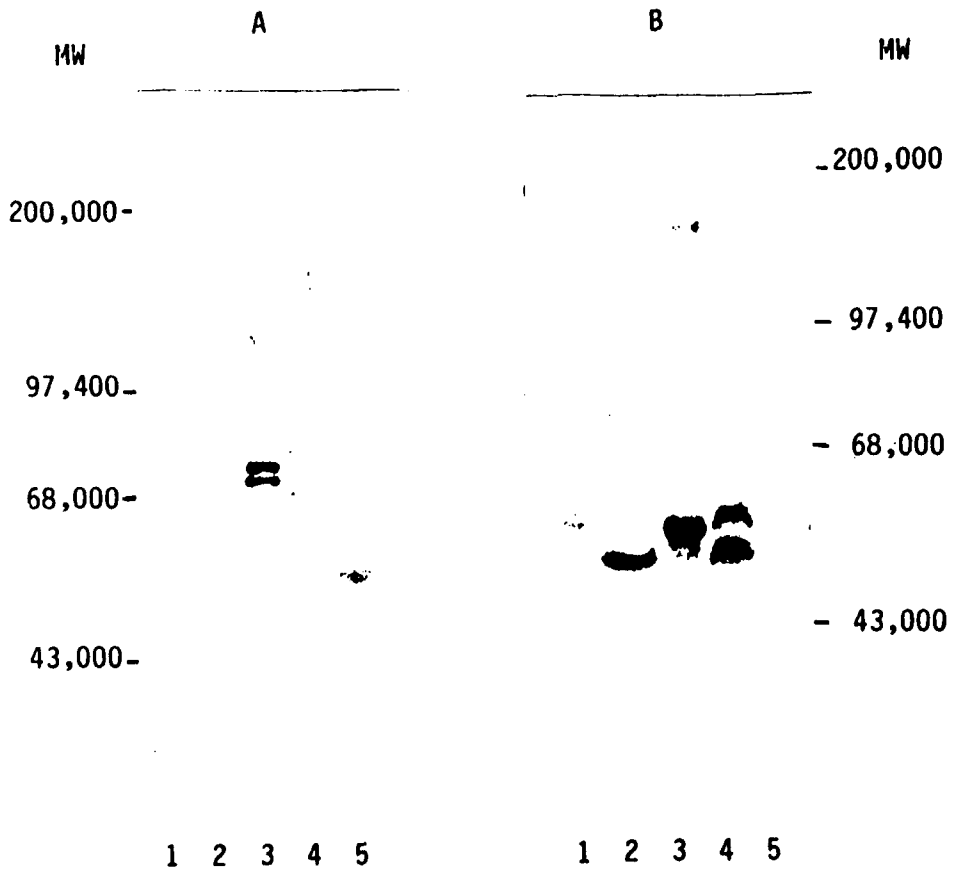

Figure 1. Western blot analysis of various OPA Anhydrase fractions. SDS-polyacrylamide gel (7.5%) were run and blotted onto nitrocellulose membrane and probed with either (A) monoclonal antibody or (B) antiserum. Lane 1 (A) and 2 (B), 25μg fraction-1 from DEAE-Sephacel step. Lane 2 (A) and 3 (B), 10μg fraction-2 from DEAE-Sephacel step. Lane 3 (A) and 4 (B), 25 μg crude enzyme extract. Lane 5 (A) and 1 (B), 20 ng purified OPA Anhydrase-2. Lane 4 (A) and 5 (B), pre-stained molecular weight markers.

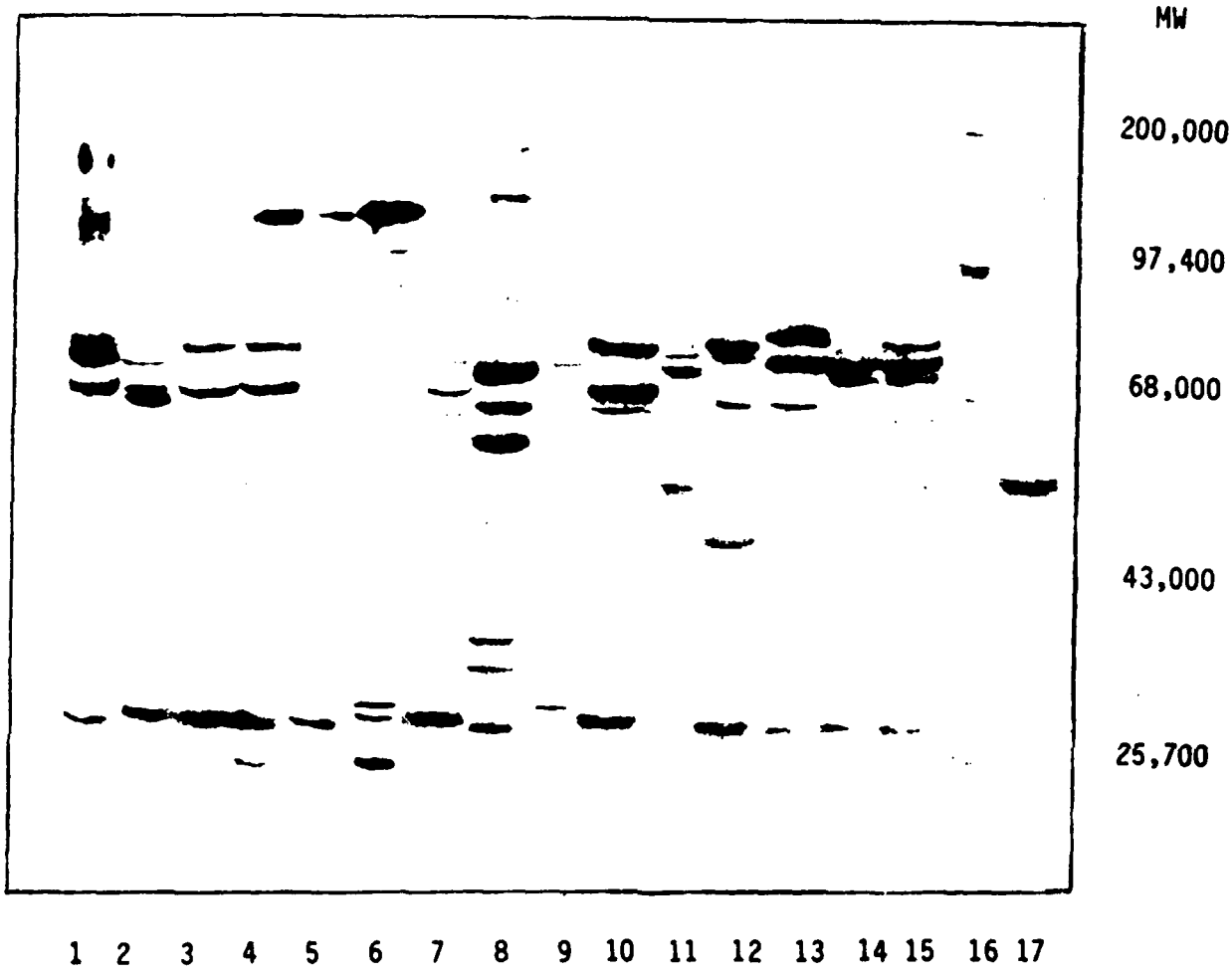

Figure 2. Western blot analysis of various *Alteromonas* species and JD-series halophiles. Lysate samples (150 μg) and purified OPA Anhydrase (50 ng) were loaded onto each lane of a SDS-polyacrylamide gel and subjected analysed for immunoreactivity with monoclonal antibody. Lane 1, *A. colwelliana*; Lane 2, *A. espejiana*; Lane 3, *A. haloplanktis(DS)*; Lane 4, *A. haloplanktis(JL)*; Lane 5, *A. luteoviolaceae*; Lane 6, *A. macleodii*, Lane 7, *A. nigrifaciens*; Lane 8, *A. putrefaciens; Lane 9, A. rubra*; Lane 10, *A. undina*; Lane 11, JD6.5; Lane 12, JD11.5; Lane 13, JD26.1; Lane 14, JD28.3; Lane 15, JD30.3; Lane 16, pre-stained protein molecular weight markers; Lane 17, purified OPA Anhydrase-2.

purified to homogeneity (16). A faint protein with similar molecular weight was also detected in this blot (lane 15). Recently, a number of Alteromonas species have been found to have low to very high enzyme activities against DFP (Table 1). When crude enzyme extracts from these strains were subjected to Western blotting and probing with Mab#6, various protein patterns were also detected (Fig. 2, lanes 1 to 10). The blot revealed that some of these Alteromonas have at least two or several different proteins recognized by Mab#6. In assays using different chromogenic substrates against OPA Anhydrases (NPEPP; p-nitrophenyl [ethyl]phenylphosphinate),phosphodiesterase (Bis; Bis[p-nitrophenyl] phosphate) and acid or alkaline phosphatase (NPP; p-nitrophenyl phosphate), various amounts of these enzyme activities were found in both JD and Alteromonas strains. For example, JD6.5 possesses high OPA anhydrase activity against DFP and NPEPP, but had little or no enzyme activities for phosphodiesterase (BIS) and phosphatase (NPP). On the other hand, A. colwelliana and A. macleodii which have very low OPA anhydrase activity showed a higher phosphodiesterase activity. The results thus suggest that these enzymes (OPA anhydrase, phosphodiesterase and phosphatase) may share some common sequences in their molecules. Since only DFP and NPEPP were used for OPA Anhydrase activity assays in this report, the possibility of Mab#6 cross-reacting with other functionally related enzymes or other unrelated cellular proteins can not be excluded.

In a similar analysis, enzyme samples from thermophile JD100, JD200 and JD300 that possess DFP-hydrolyzing activity were also screened by Mab#6. These bacteria were isolated by Dr. DeFrank from soil samples at Aberdeen Proving Ground. As shown in Fig. 3, a single band was detected by Western blotting in all three enzyme samples. No other bands were observed under the experimental conditions. The band was calculated to be approximately MW 85,000, comparable to that observed from Sephacel S-200 chromatograms with partially purified enzymes from JD100. The results further suggest that similar OPA anhydrase or related enzyme activities from other microorganisms can be recognized by Mab#6.

When equal amounts of enzyme preparations from Tetrahymena, squid, clam, hog and rabbit were analyzed and compared by Western blotting and probing with Mab#6, no bands were recognized by the monoclonal antibody (data not shown). It is unlikely that this was due to enzyme modification with carbohydrates or lipids. Generally, protein glycosylation occurs in all eukaryotic cells, the microorganisms contain few if any modifications. Cytoplasmic proteins (or enzymes) of eukaryotic cells are rarely glycosylated. Without any proven data, the results thus suggest that the antigenic domain recognized by Mab#6 in all bacterial isolates that we have studied so far is not present in these different eukaryotic cells. Another possibility is that the

TABLE 1. Comparison of *Alteromonas* Species and JD-Series Halophiles

Species	ATCC No.	Growth Medium	Protein (mg/ml)	Specific Activity (Units/mg) DFP*	NPEPP**	NPP**	Bis**
1. *A. colwelliana*	39565	PBM	16.09	0.023	0.007	0.007	0.039
2. *A. espejiana*	29659	HM	22.71	0.242	0.066	0.009	0.003
3. *A. haloplanktis*(DS)	(14393)	HM	19.45	0.417	0.096	0.009	0.001
4. *A. haloplanktis*(JL)	(14393)	HM	21.11	0.358	0.108	0.013	0.017
5. *A. luteoviolaceae*	33492	IOM	24.24	0.211	0.061	0.015	0.001
6. *A. macleodii*	27126	HM	14.96	0.002	0.005	0.001	0.040
7. *A. nigrifaciens*	19375	HM	16.48	0.168	0.057	0.013	0.001
8. *A. putrefaciens*	8071	PBM	17.25	0.071	0.021	0.010	0.018
9. *A. rubra*	29570	IOM	10.80	0.420	0.126	0.101	0.005
10. *A. undina*	29660	HM	14.50	0.688	0.158	0.048	0.001
11. JD6.5		HM	29.86	0.299	0.055	0.007	0.003
12. JD11.5 (*A. putrefaciens* ?)		HM	26.00	0.022	0.012	0.006	0.032
13. JD26.1		HM	21.48	0.094	0.016	0.078	0.003
14. JD28.3		HM	18.25	0.154	0.018	0.324	0.067
15. JD30.3		HM	19.58	0.118	0.011	0.123	0.019

* [DFP] = 3.0 mM ** [NPEPP, NPP, Bis] = 0.1 mM

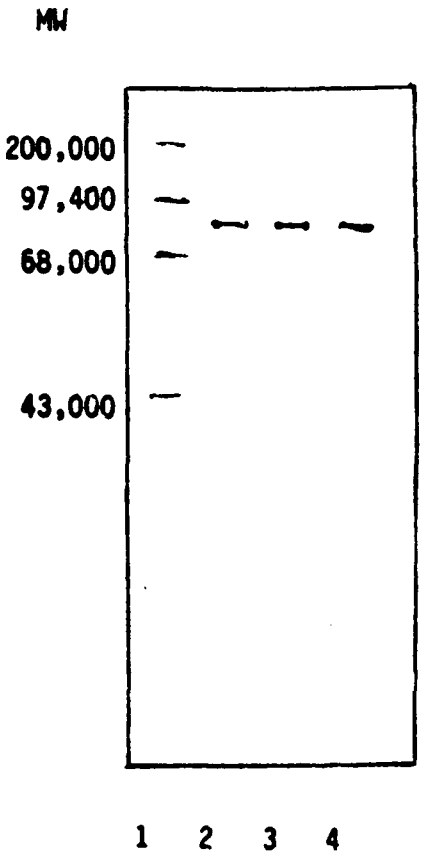

Figure 3. Western blot analysis of various JD-series thermophiles. The lysate samples (150 μg) were subjected to SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane and probed with monoclonal antibody. Lane 1, pre-stained protein molecular weight markers; Lane 2, JD100 Lane 3, JD200; Lane 3, JD300.

amounts of enzymes in these cells are too small to be detected under these experimental conditions.

4. CONCLUSION

The polyclonal anti-serum and monoclonal antibody (Mab#6) prepared was shown to react specifically with OPAA-2. Mab#6 should be useful for the cloning of this enzyme. Because monoclonal antibodies bind to only one antigenic domain, they can distinguish between closely related molecules (17-19). The principal difficulty in using monoclonal antibodies in Western blotting analysis is that many will not recognize antigenic domains that are destroyed by the denaturing reagents used in the the preparation of sample. However some monoclonal antibodies work well and give extremely clean and sensitive results.

The experiments described here were carried out in order to see whether Mab#6 can be used as probe to screen OPA anhydrases from various sources. The studies suggest that only microorganisms with high levels of OPA anhydrases can be recognized by its unique binding property. These studies do not exclude the possibility that Mab#6 might cross-react with other functionally related enzymes or totally unrelated proteins. Because the antigenic domain recognized by a monoclonal antibody is usually a relatively small protein structure which can be composed of only 4 or 5 amino acids, there is a chance that such domain can be found on another unrelated polypeptides. Therefore, any unexpected band on the immunoblot should be also treated as spurious cross-reaction until proven otherwise.

While applicable only in somewhat selective circumstances, the Mab#6 can become a powerful tool for discovering new members of OPA Anhydrases. Clearly, these prospects are only possible today because parallel development in molecular biology, molecular immunology, and instrumentation permit high volume screening with sensitivity and selectivity. Such methods will facilitate large scale searches for microbial OPA Anhydrases active against various organophosphorus compounds. For the future, we anticipate that the techniques may also be useful for the purification, detection and cloning of OPA anhydrases from all the microorganisms that we have studied in this report.

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